Evaluation of an in-house ELISA using the intermediate species *Leptospira fainei* for diagnosis of leptospirosis

Pascale Bourhy,1 Muriel Vray2 and Mathieu Picardeau1

1Institut Pasteur, Biology of Spirochetes Unit, National Reference Center and WHO Collaborating Center for Leptospirosis, Paris, France
2Institut Pasteur, Unité de Recherche et d’Expertise Epidémiologie des Maladies Emergentes, Paris, France

Leptospirosis is recognized as an emerging zoonotic disease generally affecting urban slums in developing countries and tropical regions. A combination of non-specific symptoms, low awareness among the medical community and a lack of readily available diagnostic tests have made leptospirosis an underdiagnosed disease. In this study, we tested an in-house ELISA with formalin-treated and boiled bacteria from the intermediate species *Leptospira fainei* as an antigen to detect *Leptospira*-specific IgM antibodies. A total of 819 serum samples, tested by a microscopic agglutination test (MAT) as a reference test, were used to evaluate the ELISA. Compared with positive and negative sera, the ELISA showed 94% sensitivity and 99% specificity. Positive and negative likelihood ratios were 94 and 0.06, respectively. No cross-reactivity was observed in sera from subjects with dengue and syphilis infections. The kappa value was 0.92 (95% confidence interval 0.88–0.96), which indicated excellent agreement between the MAT and ELISA. The overall performance of this in-house ELISA suggests applicability as a rapid screening test for the diagnosis of leptospirosis in resource-limited settings and in hospitals and laboratories where a MAT is not available.

INTRODUCTION

The World Health Organization (WHO) estimates that more than 1.7 million severe cases of leptospirosis occur annually with a case fatality rate of ~10% (Abela-Ridder et al., 2010). Leptospirosis is diagnosed worldwide with most of the reported cases originating from countries with tropical and subtropical climates. Leptospirosis has also been recognized as an emerging zoonotic disease. Rapid urbanization and the development of slum communities, which provide conditions for rat-borne transmission, have resulted in an increase in the number of reported cases of leptospirosis (Lau et al., 2010). The wide spectrum of symptoms of leptospirosis, ranging from a flu-like syndrome to renal failure, mimics the clinical presentations of many other diseases, including dengue fever, malaria and rickettsioses. It is therefore not always possible to reliably predict the pathogen based on clinical signs and symptoms (Levett, 2001; McBride et al., 2005). Traditionally, serological diagnosis of leptospirosis has relied on a microscopic agglutination test (MAT). However, this technique requires specific equipment and/or laboratories and highly trained staff and is therefore restricted to a few reference laboratories. IgM antibody-capture microplate ELISAs, as well as macroagglutination, immunofluorescence, indirect haemagglutination, latex agglutination and lateral flow assays, have become the tests of choice for the rapid screening of serum samples from suspected cases of leptospirosis in many laboratories (Levett, 2001; McBride et al., 2005). The performance of these tests varies in terms of sensitivity and specificity. ELISAs are widely used for the serodiagnosis of leptospirosis. ELISA can be performed with minimal training and limited equipment, and typically provides results in 4 h. In general, the antigens used for ELISA are based on the non-pathogen *Leptospira biflexa*, but these assays may not recognize the diversity of all infecting serovars, and the overall sensitivity of these tests is poor (Levett, 2001; McBride et al., 2005).

To date, 21 species and >300 serovars have been identified in the genus *Leptospira*, among which five ‘intermediate’ species (*Leptospira inadai*, *Leptospira broomii*, *Leptospira fainei*, *Leptospira woffii* and *Leptospira licerasiae*) are of unclear pathogenicity and form a group distinct from those that are known pathogens and saprophytes (Cerqueira & Picardeau, 2009). The intermediate *L. fainei* serovar Hurstbridge was initially isolated from the uteri and...
kidneys of pigs in Australia (Perolat et al., 1998) and subsequently detected by serological analysis in humans, including cases of leptospirosis, in Australia and the Seychelles (Chappel et al., 1998; Yersin et al., 1998). More recently, *L. fainei* has been isolated from patients in Denmark (Petersen et al., 2001) and France (Arzouni et al., 2002). *L. fainei* DNA was also amplified from bats and environmental water in Peru (Matthias et al., 2005; Ganoza et al., 2006). Consequently, this species can be found in humans, animals and the environment. Because of its phylogenetic position, between saprophytes and pathogens, this intermediate species may have a wider range of antigens in common with pathogenic serovars than the saprophyte *L. biflexa*. A major challenge for the serological diagnosis is to find an antigen that will allow a broad spectrum of detection of antibodies directed against the variety of serovars. We therefore decided to develop an ELISA based on a whole-cell antigen extract obtained from *L. fainei* serovar Hurstbridge.

**METHODS**

**Serum samples.** The National Reference Center for Leptospirosis, which is also a WHO Collaborating Center, at the Institut Pasteur of Paris, France, performs serological diagnosis on sera (>4000 serum samples per year) from patients from mainland France and French overseas territories (Guadeloupe, Martinique, Guyane, French Polynesia, etc.) by MAT. A total of 819 human sera were used in this study. The sera were tested at the National Reference Center for Leptospirosis for diagnostic purposes between 2010 and 2011. Four panels of human sera were used for evaluation of the ELISA.

Panel A consisted of 149 confirmed cases with clinical suspicion of leptospirosis and seroconversion between paired sera against any pathogenic serogroup by MAT (from a negative titre to a titre ≥ 400). The patients (123 males and 26 females, mean age 44 years) originated from mainland France (56), Martinique (46), Guadeloupe (42) and other countries (five) (Tables 1 and 2).

Panel B consisted of 165 probable cases with clinical suspicion of leptospirosis and a single serum sample with a MAT titre ≥ 400. The patients (137 males and 28 females, mean age 46 years) originated from mainland France (70), Martinique (45), Guadeloupe (44) and other countries (six).

Panel C consisted of 48 confirmed negative cases from healthy blood donors (Platform Investigation Clinique et Acces aux Ressources Biologiques at the Institut Pasteur, France) or from patients with evidence of recent infection for dengue (S. Guyomard, Institut Pasteur of Guadeloupe, France) and syphilis (Institut Pasteur de Paris, Center Medical, France). All negative samples were confirmed by MAT.

Panel D consisted of 154 probable negative cases with clinical suspicion of leptospirosis and MAT-negative results (titres <50) with paired sera collected at least 2 weeks apart. Negative cases (101 males and 53 females, mean age 43 years) originated from mainland France (102), Martinique (19), Guadeloupe (17), Guyane (12) and other countries (four).

This study was part of a protocol approved by the Institut Pasteur (protocol # RBM2008-16) and the French Ministry for Education & Research (protocol nos AC-2007-44 and DC-2010-1197). All sera were tested as anonymous samples.

**MAT.** All samples were characterized using a MAT as the reference test. The MAT was performed at the National Reference Center for Leptospirosis using the following antigens: serogroups Australis (strain Ballico), Autumnalis (strain Akikayami A), Bataviae (strain Van Vienen), Canicola (strain Hond Utrecht IV), Ballum (strain Castellon 3), Cynopteri (strain 3522C), Grippotyphosa (strain Moskva V), Sejroe (strains M84 and Hardo Pratijino), Hebdomadis (strain Hebdomadis), Icterohaemorrhagiae (strains Wijnberg and Verdun), Panama (strain CZ214K), Pomona (strain Pomona), Pyrogenes (strain Salinem), Tarassovi (strain Mitis Johnson) and the non-pathogen serogroup Semaranga (strain Patoc). Since January 2011, serogroups Celledoni (strain 201101963), Djasiman (strain Djasiman), Mini (strain 200801925), Sarmin (strain Sarmin), Shermani (strain 1342 K), Javanica (strain Poi) and Louisiana (strain LUC1845) have been included in the panel of antigens. The Semaranga serogroup, which belongs to a non-pathogenic *Leptospira* species, was included in our analysis because it has cross-reactivity with pathogenic serogroups and can be indicative of an infection. Sera were screened at a dilution of 1/50 and positive sera were titrated to the end point using standard methods. A high agglutination titre of the serum with one particular serogroup was taken to identify the presumptive serogroup of the infecting bacterium (Postic et al., 2000).

**Antigen preparation.** A 10 ml pre-culture of *L. fainei* serovar Hurstbridge strain BUT 6<sup>T</sup> in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) was used to inoculate 1 litre of EMJH. This culture was incubated at 30 °C for 1 week with constant shaking until reaching an absorbance value at 420 nm (A<sub>420</sub>) of ≥0.5. The culture was then left standing at room temperature for 2–4 h after the addition of 2 ml 37% formalin, and the formalin-killed bacteria were boiled for 45 min in a boiling water bath. Lastly, the pH was adjusted to 9.6 and this preparation was stored at 4 °C for 1 year. This crude preparation was used directly as antigen for the ELISA.

**In-house IgM ELISA.** Flat-bottomed polystyrene 96-well microtitre plates (Immuno1B; Dutscher Scientific) were coated for 3 days at 37 °C with 75 μl of the antigen preparation. The coated plates were stored under dry conditions at room temperature for 6 months until use.

The plates were washed three times with PBS plus 0.2% Tween 20 (PBST; pH 7.2) and the wells were blocked with 75 μl 5% non-fat milk in PBS (PBSM; pH 7.2) for 1 h at 37 °C (or alternatively overnight at 4 °C). The plates were then washed three times with PBST and incubated for 1 h at 37 °C with 75 μl of a 400-fold dilution of the serum in PBSTM. All reactions were performed in duplicate. A pool of positive sera (MAT titre ≥800) was serially diluted (1/400–1/204800) and included on each plate as an internal standard. The plates were washed three times with PBST and incubated for 1 h at 37 °C with 75 μl of a 1500-fold dilution of horseradish-peroxidase conjugated human affinity purified antibody specific to goat IgM (Bio-Rad). The

**Table 1.** Performance of the ELISA to detect pathogenic leptospiral antibodies in sera from confirmed cases of leptospirosis and from healthy blood donors and patients with syphilis and dengue using MAT as a reference standard

<table>
<thead>
<tr>
<th>MAT negative</th>
<th>MAT positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA negative</td>
<td>48*</td>
<td>8</td>
</tr>
<tr>
<td>ELISA positive</td>
<td>0</td>
<td>141</td>
</tr>
<tr>
<td>Total</td>
<td>48*</td>
<td>149</td>
</tr>
</tbody>
</table>

*Including 12 sera from dengue patients and six sera from syphilis patients.
plates were washed twice with PBST and twice with PBS, and 75 μl 2,2'-azino di(3-ethyl benzthiazoline-6 sulphonic acid) peroxidase substrate (Roche) was added per well. The plates were incubated in the dark at 37 °C for 30 min and 

A<sub>415</sub> was measured using an ELISA plate reader (Bio-Rad).

The ELISA cut-off was set at 3SD above the mean 

A<sub>415</sub> of sera from 30 healthy donors. To determine antibody titre, a pool of positive sera (MAT titre $\geq$800) was serially diluted (twofold dilutions from 1/400 to 1/204 800) and then tested on the ELISA. The end-point titre was determined by the last diluted sample that gave an 

A<sub>415</sub> value above the cut-off, therefore defining a cut-off titre of 400. A reference sample with an ELISA titre of 400 was included in each plate to evaluate possible variation in titres.

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio for a negative (LR<sup>-</sup>) and a positive (LR<sup>+</sup>) result for the assays were calculated based on negative and positive samples (Tables 1 and 2). The $\kappa$ coefficient [with 95% confidence interval (CI)] was calculated to evaluate the agreement between methodologies, using the interpretation scale of Landis & Koch (1977). The sample size was chosen as ~150 confirmed cases in order to show a sensitivity of 90% with a precision of 5%.

RESULTS

A total of 819 serum samples collected from 2010 to 2011 were selected for evaluation of the ELISA. Half of the serum samples originated from the French West Indies (Martinique and Guadeloupe) where the mean incidence of leptospirosis is $\geq$30 per 100 000 inhabitants. The remaining half were from patients from mainland France where the mean incidence of leptospirosis is approximately 0.4 per 100 000 inhabitants. A MAT, which can identify a presumptive serogroup, was used as a reference standard to evaluate the ELISA procedure described in the present study.

A diagnostic cut-off value for a positive response was set to 1/400 by testing infected and non-infected controls. Any serum sample showing a titre $\geq$400 was considered positive; below this cut-off value, results were considered to be background reactivity caused by non-specific antibodies.

The ELISA was assessed against a panel of samples including confirmed and probable cases, as well as probable negative cases and sera derived from healthy donors and from patients with infections other than leptospirosis. The results are summarized in Tables 1, 2 and 3.

For confirmed cases, the ELISA performed on convalescent sera displayed a sensitivity of 95% detecting almost all patients (141/149). In terms of specificity, the ELISA showed 100% specificity (48/48) for patients without leptospirosis, including patients with evidence of dengue
ELISA for the serodiagnosis of leptospirosis

Table 3. Performance of the developed ELISA to detect pathogenic leptospiral antibodies in sera from both probable and confirmed cases using the MAT as a reference standard

<table>
<thead>
<tr>
<th></th>
<th>MAT negative</th>
<th>MAT positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA negative</td>
<td>199</td>
<td>19</td>
<td>218</td>
</tr>
<tr>
<td>ELISA positive</td>
<td>3</td>
<td>298</td>
<td>301</td>
</tr>
<tr>
<td>Total</td>
<td>202</td>
<td>317</td>
<td>519</td>
</tr>
</tbody>
</table>

fever and syphilis (Table 1). The PPV and NPV were 100 and 86 %, respectively. Likelihood ratios, which are clinically more useful than sensitivity and specificity for reporting the performance of diagnostic tests, were also calculated. The LR+ of the ELISA was 94, meaning that a MAT-reactive serum sample is 94 times more likely to have a positive test than a person without the disease. The LR− (people who are disease negative and test negative) was 0.05. Agreement between the ELISA and MAT was excellent, with a κ score of 0.90 (95 % CI 0.82–0.98).

In this study, we also included probable cases of leptospirosis in evaluation of the ELISA. Combining the probable and confirmed cases (202 MAT-negative samples and 317 MAT-positive samples) gave an overall sensitivity and specificity of 94 and 99 %, respectively (Table 3). The ELISA gave a similar PPV and NPV (91 and 99 %, respectively) and likelihood ratios (LR+ 94 and LR− 0.06) in comparison with the results obtained for confirmed cases. The κ value was 0.92 (95 % CI 0.88–0.96), showing excellent agreement between the two assays.

According to the MAT titres, serogroup Icterohaemorrhagiae was the predominant serogroup in our samples and accounted for 139/317 cases (44 %). Other serogroups included Grippotyphosa (n=32), Ballum (n=17), Hebdomadis (n=12), Canicola (n=11), Sejroe (n=9), Australis (n=7), Tarrassovi (n=6), Bataviae (n=4), Pyrogenes (n=4), Autumnalis (n=3), Cynopteri (n=3), Pomona (n=3), Mini (n=1), Panama (n=1) and Sarmin (n=1). The probable infecting serogroup could not be assessed for 64 patients due to the presence of coagglutinins in their sera. The ELISA was able to detect antibodies against all these serogroups in the sera from patients originating from mainland France (n=127) and the French West Indies (n=190). The 19 ELISA-negative patients (Table 3) were MAT positive for serogroups Grippotyphosa (n=10), Australis (n=3), Ballum, (n=2), Panama (n=1) and Icterohaemorrhagiae (n=1); the serogroup could not be assessed for two sera due to the presence of coagglutinins.

Among the 149 confirmed cases of leptospirosis diagnosed by MAT seroconversion on paired sera, the first serum samples, which were negative by MAT, were analysed by ELISA. A total of 66 samples (44 %) were positive by ELISA. The others remained seronegative by ELISA. The ELISA therefore had a much higher sensitivity than the MAT during the acute phase of the infection, enabling the early detection of leptospirosis.

DISCUSSION

Leptospirosis is under-reported in many countries due to a lack of diagnostic techniques. The initial diagnosis must rely on the epidemiological history (details of possible occupational or recreational exposure) and clinical signs and symptoms, but because there is much overlap in the clinical presentation of undifferentiated febrile illnesses, laboratory confirmation is essential. Early laboratory diagnosis is important to provide appropriate treatment of patients and to take rapid measures in case of an outbreak. Unfortunately, few tests possess both high sensitivity and specificity; this is particularly true during the acute phase of the disease (Levett, 2001; McBride et al., 2005).

The reference method for diagnosis of leptospirosis is the MAT. However, this test is fastidious to perform and to interpret. A MAT involves mixing serial dilutions of sera with live strains that are representatives of the major Leptospira serogroups. After incubation, agglutination of the leptospires by the patient’s or a control serum is observed by dark-field microscopy.

A recent meta-analysis on IgM ELISAs (55 studies) reported an overall sensitivity and specificity of 80 and 94 %, respectively (Signorini et al., 2012). Our ELISA was highly sensitive (94 %) and specific (99 %). Reported variations in the performance of ELISAs may be due to several factors, including the type of antigen used in the test, the selected cut-off value, the choice of the case definition and population-related differences (Limmathurotsakul et al., 2012; Signorini et al., 2012). Participation in a programme of interlaboratory comparison or proficiency testing of ELISA tests such as the one established for MATs (Chappel et al., 2004) should help in determining factors responsible for the variations found in ELISAs.

Our ELISA was able to detect anti-Leptospira antibodies from a wide range of pathogenic serogroups, indicating that the assay should recognize the diversity of circulating strains. The assay was also able to detect IgM antibodies during the acute phase, when agglutinating antibodies were not detectable by MAT. The ability of ELISA to detect anti-Leptospira antibodies earlier in the course of the disease than MAT has already been largely recognized (Winslow et al., 1997; Brandão et al., 1998; Cumberland et al., 1999; Levett et al., 2001; Bajani et al., 2003; Chirathaworn et al., 2007; Doungchawee et al., 2008; Aviat et al., 2010).

ELISA results give no indication of the infecting serovar, and confirmatory diagnosis of leptospirosis should be performed by isolation or nucleic acid detection, or by seroconversion or a fourfold rise in MAT antibody titre with paired sera in patients presenting with signs and symptoms that are consistent with leptospirosis. The ELISA can therefore be used as a simple and rapid laboratory
screening test for the diagnosis of leptospirosis when antibody titres are increasing and leptospires have been cleared from the blood. Thus, PCR on blood samples would not be a suitable method of detecting leptospires when antibody titres are high. As IgM antibody usually persists for >5 months (Silva et al., 1995), ELISA can also be used for several months after the onset of symptoms.

It can also be used as an effective tool for early outbreak warnings and surveillance in resource-limited settings. A patent related to this antigen has been deposited (Bourhy et al., 2012). Further application of this antigen may include the generation of a dipstick assay as a rapid diagnostic test as an IgG ELISA that could be used for seroprevalence studies.

ACKNOWLEDGEMENTS

We thank the technicians from the National Reference Center for Leptospirosis (Sylvie Brémont, Annie Landier and Farida Zinini) for serological analysis of sera. This work was funded by the Institut Pasteur and the French Ministry of Health (InVS).

REFERENCES


